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The length and location of CAG trinucleotide repeats in the androgen receptor N-terminal domain affect transactivation function

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ABSTRACT

Some transcription factors contain stretches of polyglutamine encoded by repeats of the trinucleotide CAG. Expansion of the CAG repeat in the androgen receptor (AR) has been correlated with the incidence and severity of X-linked spinal and bulbar muscular atrophy (Kennedy's disease). In order to understand the relationship of this mutation to AR function, we constructed ARs that varied in the position and size of the polyglutamine tract, and assayed for the abilities of these mutant receptors to bind androgen and to activate transcription of several different AR-responsive reporter genes. Elimination of the tract in both human and rat AR resulted in elevated transcriptional activation activity, strongly suggesting that the presence of the polyglutamine tract is inhibitory to transactivation. Progressive expansion of the CAG repeat in human AR caused a linear decrease of transactivation function. Importantly, expansion of the tract did not completely eliminate AR activity. We postulate that this residual AR activity may be sufficient for development of male primary and secondary sex characteristics, but may fall below a threshold level of activity necessary for normal maintenance of motor neuron function. This functional abnormality may be representative of other genetic diseases that are associated with CAG expansion mutations in open reading frames, such as spinocerebellar ataxia type I and Huntington's disease.

INTRODUCTION

Homopolymeric stretches of amino acids are found in an increasing number of proteins (1). Polyglutamine tracts of twenty or more residues are present in at least 33 transcription factors, including the *Drosophila* Antennapedia (2), engrailed and Notch proteins (3), human TATA binding protein (4,5), yeast GAL11(1), and the androgen receptor (AR) from several species (6–9). AR belongs to a large family of ligand-activated transcription factors that also includes the receptors for glucocorticoids, progesterone and mineralocorticoids. Each of these proteins possesses an N-terminal modulatory domain, a zinc-finger-containing DNA binding domain, and a C-terminal hormone binding domain (10). The modulatory domains of human, mouse and rat AR contain a polyglutamine tract, although the repeat in the human AR has a different location within the N-terminus than in the mouse and rat receptors. Interestingly, the N-terminal domain of the rat glucocorticoid receptor (GR) has a tract whose size is variable, ranging from 7 to 21 glutamines in length (11), while the tracts in mouse and human GRs have only 9 and 2 residues, respectively (12,13).

Polyglutamine tracts are often encoded by repetition of the codon CAG, forming a trinucleotide repeat or microsatellite. Several human genetic diseases have recently been associated with CAG trinucleotide repeat expansion (14). X-linked spinal and bulbar muscular atrophy (Kennedy's disease) (15), Huntington's disease (16), and spinocerebellar ataxia type 1 (SCA1) (17) have all been associated with this type of genetic alteration. Huntington's disease, SCA1, and Kennedy's disease share several features: (1) the diseases are late-onset, neurological disorders, with larger repeats correlated with earlier age of onset; (2) the CAG repeats are present in putative open reading frames and encode polyglutamine; and (3) the size range of the repeat in affected individuals is approximately twice the range in the normal population. In Kennedy's disease, a degenerative neuromuscular syndrome (18,19), the expanded CAG trinucleotide repeat is in the AR N-terminal domain, and the size of the repeat is inversely correlated with the severity of the symptoms (20–24). Because the mutations associated with these three diseases are so similar, it is possible that studying the effect of CAG expansion on AR function may reveal a common underlying mechanism that results in the disease phenotypes in Huntington's disease and SCA1 (25,26).

Several naturally occurring mutations in AR are known to result in loss of activity and lead to disease. Point mutations in the AR hormone- or DNA-binding domains are a common cause of complete androgen insensitivity syndrome in males (27). A

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second kind of loss-of-function mutation in AR has been described in testicularly feminized (*Tfm*) mice, in which a truncated, inactive AR protein lacking the entire N-terminal transactivation domain is synthesized as a result of translational re-initiation (28). The expansion of the CAG trinucleotide repeat in the N-terminal domain represents a third type of genetic alteration in AR that is associated with disease. However, this particular mutation does not seem to result in total loss of AR function, as individuals with Kennedy's disease are phenotypic males who show only slight signs of androgen insensitivity (29).

Recently, a study of androgen-mediated feedback inhibition of pituitary, hypothalamic, and testicular hormones in 26 patients with Kennedy's disease concluded that the mutant ARs suppressed hormone synthesis less well than the wild-type AR in control patients (30), suggesting decreased AR activity in patients with Kennedy's disease. Consistent with this observation, Mahtre *et al.* (26) have reported that ARs from two Kennedy's disease patients have reduced transcriptional activation function. However, in this study the decreased activity was difficult to quantitate, and therefore could not be correlated to CAG repeat length. In addition, there were no data to address the mechanism for the effects of CAG repeat expansion on AR function.

To better understand the relationship of the polyglutamine tract to AR transactivation function, we constructed AR derivatives that varied in the presence, position, or size of the CAG repeat. These mutant receptors were biochemically characterized and tested for transcriptional regulatory activity. Analysis of these AR variants revealed a relationship between trinucleotide repeat length and protein function, and led to the proposal that cell-specific androgen responses may be sensitive to subtle changes in AR activity levels.

MATERIALS AND METHODS

Construction of reporter and receptor expression plasmids

The AR mutant Δ168−221 was created by digesting rat AR with *Avr*II and *Nae*I, followed by the removal of the overhang with Klenow and ligation to produce an in-frame deletion. The human/rat AR chimeric receptor AR-hKB was made by interchanging the *Kpn*I−*Bfr*I fragment from human AR with the corresponding region in rat AR. All constructs were verified by DNA sequencing and subcloned into p6R (31). Human AR polyglutamine expansion mutants were constructed as follows. The *Eag*I−*Stu*I fragment of hAR was subcloned into the *Eag*I and *Eco*RV sites of pSK$^+$ (Stratagene) to create pSKhAR-ES. The T3 primer and primer hAR-Q_3, (CCCTGCAGGGGGCT-AGTCTCTTGCTG), which contains a *Pst*I site, were used to PCR amplify a 265 base pair product, which was then cut with *Pst*I. The 93 base pair *Pst*I−*Pst*I fragment, which codes for the amino acids $LQ_{(25)}$ETSPL, was inserted into the *Pst*I fragment of pSKhAR-ES and transformed into SURE cells (Stratagene). Several of the inserts in the resultant plasmids contained fewer trinucleotides, and one plasmid contained three inserts. The *Eag*I−*Bfr*I fragments of these clones were purified and inserted into the *Eag*I and *Bfr*I sites of 6RhAR to create $6RhARQ_{35}$, $6RhARQ_{49}$, and $6RhARQ_{77}$. One clone had lost the insert as well as the endogenous glutamine tract, deleting amino acids 59−89 of the wild-type hAR, yielding $6RhARQ_1$. Before transfection, these plasmids were CsCl-purified and subjected to dideoxy sequencing using a primer containing the sequence TC-AGCTGCCCCATCCACGTTGTCCC (+465 to +489). All AR derivatives were subcloned into pCMX (32) for transfections used in ligand-binding assays.

The pMMCAT reporter plasmid contains the mouse mammary tumor virus (MMTV) long terminal repeat pstream of the chloramphenicol acetyltransferase (CAT) gene (33). The TAT3CAT reporter gene contains three tandem copies of HREII from the tyrosine amino transferase gene linked upstream of the minimal *Drosophila* alcohol dehydrogenase promoter at the −33 TATA element (34). SLPCAT refers to the C'D2 reporter gene derived from the sex-limited protein complex androgen-dependent enhancer (35).

Cell culture and transfections

Monkey kidney CV-1 and COS-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% defined calf bovine serum (CBS). For transactivation assays, 1×10^6 CV-1 cells were transfected by the calcium phosphate method (36) using ~10 μg each p6R receptor expression plasmid (normalized for the size of the receptor), 2 μg pMM-CAT reporter plasmid, 5 μg β-galactosidase expression plasmid pEQ176 (37), and sheared calf thymus DNA up to 25 μg per 10 cm^2 plate. For each ligand-binding assay and Western blot, four 15 cm^2 plates containing 2×10^6 COS-7 cells in DMEM plus 5% charcoal-stripped CBS were transfected as above, except that ~30 μg each pCMX receptor expression plasmid (normalized for the size of the receptor) and 1 μg pRSCAT (38) were used per plate, and the DNA precipitates were left on the cells for 16 h.

CAT and ligand-binding assays

Forty hours after transfection, the cells were harvested and assayed for CAT activity (33) following a normalization for transfection efficiency using β-galactosidase (β-gal) activity (39). All transfection experiments were performed at least seven independent times, and the percent conversion of chloramphenicol to the acetylated forms was quantitated by Betascope (Betagen) analysis. CAT activity is expressed as % conversion/β-gal unit/h CAT assay. β-gal units are defined as the A_{410}/mg protein/min β-gal assay, multiplied by the μg protein in the CAT assay. Statistical analysis was performed using a two-group paired two-tail *t*-test.

The dissociation constants (K_ds) for the synthetic androgen methyltrienolone (R1881) were determined by pooling the four 15 cm^2 plates of transfected COS-7 cells, preparing cell extracts by sonication, and measuring the amount of receptor-specific radiolabeling using [^{3}H]R1881 (38). Final concentrations of [^{3}H]R1881 ranged from 0.375 to 30 nM, and non-specific binding was measured by including 1000-fold molar excess of unlabeled R1881. A portion of the cell extract was removed before the ligand binding assay and reserved for detection of AR protein by Western blotting.

Western blots

An AR C-terminal segment encoding amino acids 639−902 was expressed and purified from *E.coli* strain BL21 (DE3) using the pET3A vector (40) (Stephen E.Rundlett and R.L.Miesfeld, unpublished). Anti-AR polyclonal antiserum (ARb) was collected from two rabbits injected with the purified fusion protein, and used at a 1:1000 dilution for detection of AR derivatives by Western blot analysis.

β-Mercaptoethanol and sodium dodecyl sulfate (SDS) were added to transfected COS-7 cell extracts to 1% final

concentration, and 75 μg protein were subjected to electrophoresis on a 7.5% polyacrylamide (37.5:1)−SDS gel. Proteins were transferred to Zeta-Probe membrane (BioRad) by electroblotting at 60 V for 5 h at 4°C. Non-specific sites were blocked for 1 h in powdered milk, and AR derivatives were detected using anti-AR rabbit polyclonal antisera (ARb2), followed by incubations with biotinylated anti-rabbit secondary antibody (BioRad) and avidin-conjugated horseradish peroxidase (Pierce). The membrane was incubated with peroxidase substrates hydrogen peroxide and ImmunoPure AEC (Pierce) to visualize the bands.

RESULTS

To test whether CAG repeats are inhibitory to AR transactivation function, we took advantage of the fact that, although both human and rat wild-type AR contain long tracts of CAG repeats, they are localized to different regions within the N-terminal domains (7,41). Figure 1A shows the structures of several AR derivatives, including a rat AR mutant in which the region containing the 22 endogenous CAG repeats was deleted (Δ168−221).

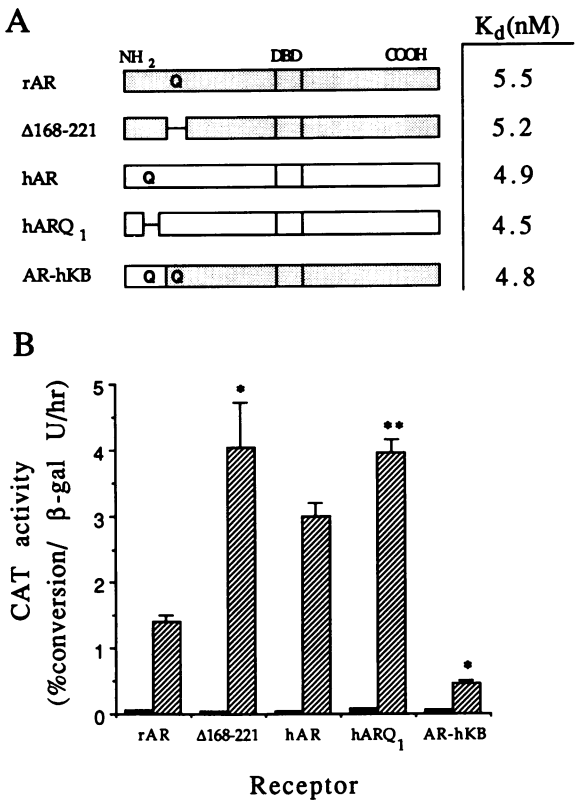

Figure 1. Presence of polyglutamine tract decreases AR transactivation function. (**A**) Structure and dissociation constants (K_ds) of human and rat AR derivatives. Sequences from rat (r) and human (h) AR are designated by open and stippled boxes, respectively. The amino (NH_2) and carboxy (COOH) termini are labelled and the relative position of the DNA-binding domain (DBD) is shown. The polyglutamine tracts are denoted as Q. The K_ds were determined by measuring the amount of receptor-specific radiolabelling using concentrations of [^{3}H]R1881 ranging from 0.375 to 30 nM. (**B**) Transcriptional transactivation activities. Activities of the AR derivatives using the reporter pMM-CAT in CV-1 cells in the absence (black bars) and presence of 1 μM dihydrotestosterone (hatched bars) are shown. Error bars represent the standard error of the mean (SEM) of at least 7 experiments. *P < 0.002 for Δ168−221 or AR-hKB compared to rAR; **P < 0.02 for $hARQ_1$ compared to hAR.

Transactivation assays in transfected CV-1 cells with MMCAT demonstrated that removal of the CAG repeat in rat AR caused a 3-fold increase in transactivation (Figure 1B). Similarly, deletion of the 25 residue endogenous repeat in human AR resulted in higher transactivation (compare hAR to $hARQ_1$), suggesting that a polyglutamine tract in either position is inhibitory. This conclusion was supported by transfection assays using AR-hKB, a receptor that contains polyglutamine tracts in both the human and rat AR locations, and that exhibited a transactivation function significantly lower than either wild-type receptor. [^{3}H]R1881-binding assays revealed similar hormone binding characteristics for each receptor (Figure 1A) and Western blot analysis using anti-AR antibodies showed no significant difference in the steady-state levels of protein (data not shown). Taken together, these results strongly suggest that polyglutamine tracts themselves are inhibitory to the transactivation function of AR.

Based on the observation that expansion of CAG repeats in human AR is associated with Kennedy's disease (15), and that addition of the human AR CAG repeats to the rat AR N-terminus (AR-hKB) resulted in a receptor that was less active than wild-type rat AR, we examined the effect of CAG repeat expansion on human AR transactivation function directly. Our strategy for constructing such a receptor involved PCR amplification of the entire trinucleotide repeat region, including several 3′ codons

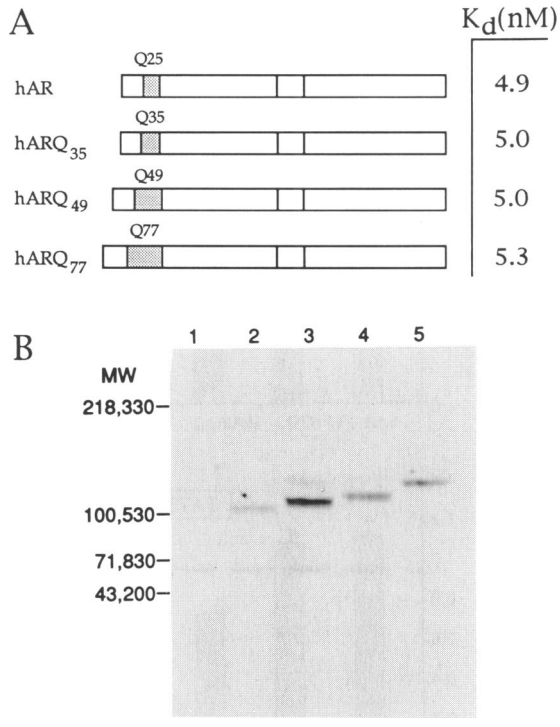

Figure 2. Characterization of the AR trinucleotide expansion mutants. (**A**) Structure and dissociation constants (K_ds) of the trinucleotide expansion mutants. The length of each polyglutamine tract is indicated by the stippled box. [^{3}H]R1818 binding assays were carried out as in Figure 1. (**B**) Western blot showing the apparent molecular weights of hAR trinucleotide repeat expansion mutants in transfected COS-7 cells. Lane 1, untransfected COS-7 extract; lane 2, hAR; lane 3, $hARQ_{35}$; lane 4, $hARQ_{49}$; and lane 5, $hARQ_{77}$. The slight difference in band intensity (the hAR band is less intense than the corresponding mutant bands) does not correlate with decreased transactivation function and therefore is likely due to small variations in transfection efficiencies.

(to serve as specific priming sites outside the repeat). Unexpectedly, DNA sequencing revealed that the resulting plasmids contained an array of CAG repeat lengths (data not shown), presumably due to recombination events that occurred during propagation in bacteria. Figure 2 shows the data from ligand-binding assays and Western blots demonstrating that the dissociation constants for the Q_{35}, Q_{49} and Q_{77} mutants are all similar to wild-type AR and that the proteins are of the expected molecular weights, respectively. These ligand-binding data are in agreement with a report that demonstrated that hAR containing 40 or 50 glutamines in the tract retained normal androgen-binding kinetics (26).

The natural AR target genes in human motor neuron cells are presently unknown and most of the AR-selective target genes that have been identified are prostate cell-specific (42,43). Therefore, to test if trinucleotide repeat expansion has any affect on AR function within different promoter contexts, we had to analyze the transactivation functions of the mutants using previously characterized AR-responsive reporter genes. The three reporter genes used for these experiments all contained similar canonical hormone response elements (HREs), but each was linked to different promoters. MMCAT contains the 1.4 kb MMTV LTR which includes the MMTV promoter, TAT3CAT has a triplicate HRE linked to a minimal promoter at the TATA element, and SLPCAT contains the complex androgen-dependent enhancer of the sex-limited protein gene upstream of the thymidine kinase promoter (35). Figure 3 shows results from multiple transfection assays using the MMCAT and TAT3CAT reporter genes. When the number of CAG repeats increased from 25 (wild-type) to 35, 49 or 77 in the mutants, we observed a progressive decrease in the level of AR transactivation function with increasing size of the trinucleotide repeat using both reporter genes (Figure 3A and 3B). The data with the SLPCAT reporter gene were more difficult to interpret because the basal promoter activity was 70% of the level of androgen-induced activity (data not shown). It may be that the SLP AR-responsive region is not sensitive to trinucleotide repeat expansion in AR, or more likely, that the fold-induction of SLPCAT by androgen was too low in these experiments to observe a statistical difference.

The MMCAT and TAT3CAT data are plotted in Figure 4 to show the relationship between CAG repeat length and AR activity. Note that there is a high correlation coefficient with a linear decline in activity as the number of repeats increases from 25 to 77.

DISCUSSION

We have constructed several AR mutants that vary in the position or size of the CAG repeat. We tested each mutant for its expression level, hormone-binding properties and transcriptional activation activities by transfection into COS-7 or CV-1 cells, monkey kidney cell lines widely used in the study of steroid receptors. The effects of the polyglutamine tract on AR function are clearly demonstrated by the mutant receptors in Figures 1 and 3. Elimination of the tract ($\Delta 168-221$ and $hARQ_1$) results in a receptor with increased activity. Conversely, expansion of the tract leads to progressive loss of transactivation. Taken together, these data strongly suggest that the polyglutamine tract, encoded by the trinucleotide repeat, is inhibitory to AR transcriptional activation function.

The polyglutamine tract is located upstream of the activation domain in hAR (44,45), but within the activation domain in rat AR (46). Indeed, we have mapped two small regions on either side of the polyglutamine tract in rat AR which are necessary

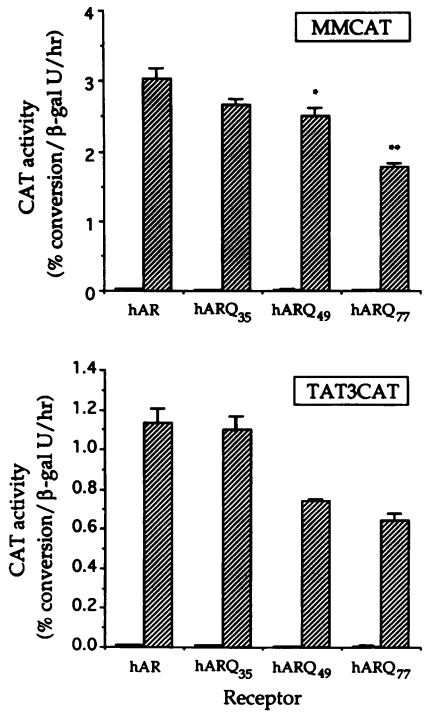

Figure 3. Expansion of the trinucleotide repeat in human AR inhibits transactivation. Transcriptional transactivation activities of the expansion mutants tested with the MMCAT (**A**) and TAT3CAT (**B**) reporter plasmids. Transfections were performed as in Figure 1B and the CAT activities obtained from cells treated without (black bars) or with 1 μM dihydrotestosterone (hatched bars) are shown. Error bars represent the SEM of at least 7 experiments. $*P < 0.02$; $**P < 0.001$.

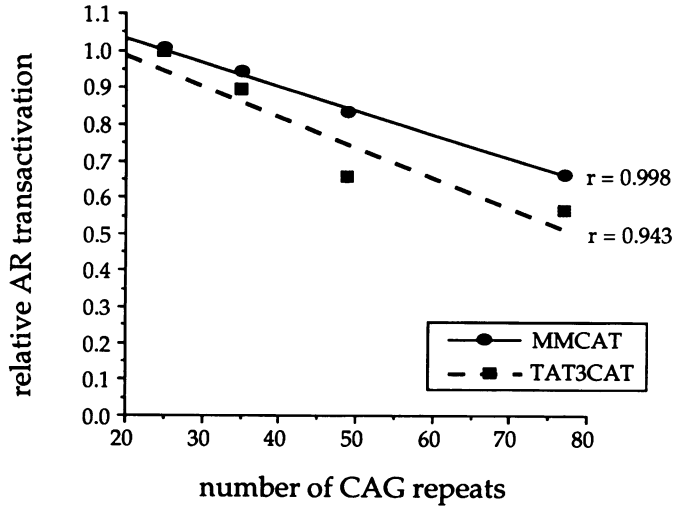

Figure 4. Transactivation activity of human AR is inversely correlated to CAG repeat length. The CAT activities of the trinucleotide repeat expansion mutants from Figure 3A (MMCAT; solid line) and 3B (TAT3CAT; dashed line) were divided by the activity of hAR, and plotted vs. the total number of CAG repeats. The data were subjected to a linear regression analysis, and the correlation coefficient, r, is shown.

for full transactivation function (N.L.Chamberlain, E.D.Driver and R.L.Miesfeld, in preparation). The difference in location of the tract relative to the activation domain may account for the observed difference in activity of the human and rat receptors (Figure 1). The polyglutamine tract in rat GR is also present just N-terminal to the mapped activation domain (47,48). The relative lack of effect of eliminating this region, either by deletion analysis (49) or in the endogenous tract in human GR, supports the notion that the juxtaposition of the tract and the activation domain may be critical. The polyglutamine tract may therefore have an indirect effect on AR function by causing structural perturbations within the transactivation domain. Alternatively, the glutamine residues may contact another protein and inhibit interactions of the activation domain with its target protein(s) by either competition or steric hindrance. The inhibition could also result from a direct interaction of the glutamine residues with a specific repressing protein.

Schaffner and colleagues have recently shown that polyglutamine can act as an activation domain when fused to the heterologous DNA binding domain of the yeast Gal4 protein, using particular reporter genes (1), but not others (50). Since activation of transcription is due to interaction of the activator and other proteins (either the basal transcription machinery or adaptor proteins) (51), it is likely that polyglutamine can be a protein interaction motif. The observation that polyglutamine tracts can either inhibit or stimulate transcription, depending on the cell and target gene contexts, has been suggested for other transcription factor motifs (3,52–54)

There are mechanisms other than direct protein interaction that account for the inhibitory nature of the polyglutamine tract in AR. Green (25) has recently proposed that the expanded polyglutamine tracts present in the proteins encoded by the genes involved in Huntington's disease, SCA1 and Kennedy's disease may be subject to transglutaminase-catalyzed cross-linking, resulting in the loss of functional protein. Mapping studies using other codon repeats and different proteins could be used to distinguish among these models.

Although there was a strong correlation between CAG repeat length and loss of AR transactivation function, it is important to recognize that even the mutant that contains a repeat three times the normal size retains approximately 60% of wild-type AR activity (Figure 4). This observation may be directly relevant to the etiology and tissue specificity of Kennedy's disease. Androgens are required for normal male development of motor neurons in the rat spinal nucleus of the bulbocavernosus (55,56) and for regeneration of facial motor neurons in rats and hamsters (57,58). Therefore, continual AR function may be necessary for maintenance of normal motor neuron function.

The ARs in the male patients with this syndrome have CAG repeats with a size range of 40–62 (24) (the range in the normal population is 11–31; 59). Extrapolating from our results in Figure 4, the levels of AR transactivation function in individuals with Kennedy's disease should be 10–30% lower than wild-type AR. The finding that the CAG repeat length in unaffected individuals does not overlap with the range of lengths in patients with Kennedy's disease suggests that a minimum threshold level of AR activity is required for normal androgen responses within motor neurons. The notion of threshold effects as a regulatory mechanism to modulate cell-specific gene expression is well documented in *Drosophila* embryogenesis (60) and in glucocorticoid induction of thymocyte apoptosis (37). In addition, age-dependent decreases in AR expression (61) and in plasma testosterone levels (62) would exacerbate the effect of decreased AR function in Kennedy's disease patients, possibly accounting for the late onset of the syndrome. Therefore even a small decrease in AR transactivation function could be sufficient to cause the disease phenotype.

We postulate that the residual level of AR activity is sufficient to ensure normal development of male primary and secondary sexual characteristics, as evidenced by the fact that affected individuals are often fertile males, and not testicularly feminized. The subtle decline of AR transactivation activity may, however, eventually lead to loss of integrity of certain tissues that require continuous high levels of AR activity, such as spinal and bulbar motor neurons. Alternatively, the effect of polyglutamine expansion may be gene-specific. For example, the activity of the mutant ARs may be compromised on genes necessary for normal neuron functions, but may be unaffected on genes involved in sexual differentiation. Finally, the activities of wild-type and mutant ARs could be cell-specific, as different sets of proteins may interact with receptors in different cell types.

It is possible that our finding that only minimal decreases in protein function may be correlated with disease manifestation could be relevant to understanding the pathophysiology of SCA1 and Huntington's disease as well.

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